Interaction Of Aziridines With Cellular Components

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INTERACTION OF AZIRIDINES WITH CELLULAR COMPONENTS

Jerry B. Stokes [1/]

ABSTRACT

Many researchers have examined the mode of action of alkylating agents, i.e., sulfur and nitrogen mustards, sulfate esters, and aziridines, and have collected data which support the interaction of alkylating agents with cellular components. In most cases, these data support a molecular attack of deoxyribonucleic acid by nitrogen and sulfur mustards, and sulfate esters, but not by aziridines. The reported interactions of these agents with deoxyribonucleic acid are compared and the experimental data and conclusions are reviewed. Also, interactions of alkylating agents with cellular components other than deoxyribonucleic acid are reviewed, and areas for future studies on the mode of action of aziridines on a molecular level are proposed.
KEYWORDS: aziridine, alkylating agent, DNA alkylation, DNA phosphate alkylation, base alkylation, crosslinking, N-7 guanine

INTRODUCTION

During the past 30 years much work has been done on the mechanism of action of biologically active alkylating agents, and the results of this work have made evident the extreme complexity of the topic. Nitrogen mustards dominate much of the work on biological effects of alkylating agents, with other compounds belonging to the same class--e.g., aziridines, sulfonic acid esters, and epoxides--appearing less frequently. Nevertheless, broad generalizations about the biological effects and biochemical mechanisms of all biological alkylating compounds have been and still are frequent.

During World War I and the postwar period, workers (Lynch et al. 1918, Pappenheimer and Vance 1920, Flury and Wieland 1921) investigating the vesicant properties of mustard gas realized that it and related chemicals possessed unusual biological properties. Marshall (1919) proposed that the action of mustard gas was due to its rapid penetration into the cell, hydrolysis within the cell to generate hydrochloric acid, and subsequent destruction of some mechanism or part of the cell by hydrochloric acid. However, Peters and Walker (1923) demonstrated that there was no correlation between the rates of liberated acid and the vesicant action of mustards. In the midthirties,

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several workers showed that sulfur mustard had antitumor effects in experimental animals; subsequently it was used clinically in the treatment of a solid tumor by direct injection into the tumor (Adair and Bagg 1931, Berenbaum 1935).

Following the original use of mustards as vesicants, biological properties were further explored, but the extreme toxicity of these agents hampered their usefulness. Therefore, the synthesis and testing of less vesicant analogs, and consequently less toxic analogs, continued during the early 1940's. The demonstration that these agents had selective effects on lymphoid tissue and other rapidly proliferating cells led to preliminary experiments to investigate possible antitumor properties. Clinical testing revealed that certain nitrogen mustards had a useful effect in the treatment of Hodgkin's disease and certain lymphosarcomas (Gilman and Philips 1946). Encouraged by these results, others investigated the biological properties of alkylating agents and their mechanism of action. Although considerable progress in understanding the mode of action of a few specific compounds has been made since the 1940's, a unified theory explaining the biological and physiological activity of all alkylating agents at the molecular level has yet to be proposed.

In this review, the primary objective will be to examine the available literature for data relevant to the mechanism of action of aziridines. The existing references dealing directly with aziridines will then be reviewed critically. The review addresses the question of whether the available experimental data justify the frequently made conclusion that the mechanism of action of aziridines is the same as that of other alkylating agents for which there is adequate evidence supporting a hypothesis.

The nomenclature of many biological alkylating agents is complex and confusing. Because common names, trade names, chemical names, and other designations have undergone many changes in the past, the common names mentioned in The Merck Index (1976, 9th edition) will be used in this review. Table 1 shows the synonyms and structures pertaining to such compounds.

BIOLOGICAL ALKYLATING AGENTS

Mechanisms of Akylation

Many articles (Ross 1953, 1962, Price 1958, Warwick 1963, Price et al. 1969) have reviewed the reaction mechanisms of alkylating agents and have proposed two mechanisms: the unimolecular mechanism (S_N1), in which the preliminary fission of a covalent bond is the rate-determining step; and the bimolecular mechanism (S_N2), which involves the attack of a nucleophilic (electron-rich) center upon the electrophilic alkylating agent. In the S_N1 mechanism the complete separation of the alkyl carbonium ion R^+ from Y^- proceeds by a slow rate-determining process that is enhanced by a polar solvent.

Table 1.--Widely used alkylating agents

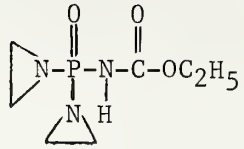
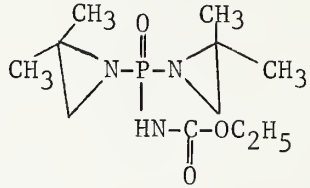
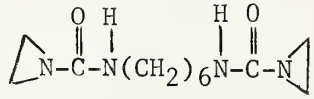
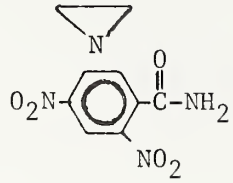
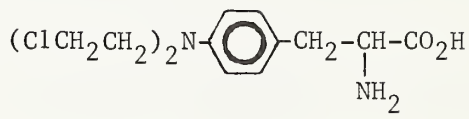
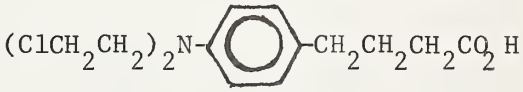
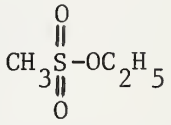
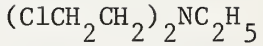
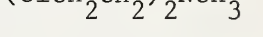
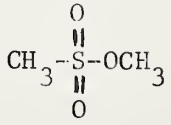
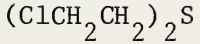
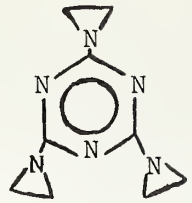
Common name	Other names, abbreviations, identification number	Structure
AB-100	Ethyl[bis(1-aziridinyl)phosphinyl]carbamate; ethyl N-[bis(ethyleneimido)phosphoro]carbamate; bis(ethylenimido)phosphorylurethane; urethimine; Uredepa; Avinar; AI3-50450; NSC-37095	
AB-132	Ethyl[bis(2,2-dimethyl-1-aziridinyl)phosphinyl]= carbamate; ethyl N-[bis(2,2-dimethylethylenimido)= phosphoro]carbamate; Meturedepa; Turloc; AI3-50425; NSC-51325	
Ethylenimine	Aziridine; AI3-50324	
CB 1263	N,N'-1,6-hexamethylenebis(1-aziridinecarboxamide); AI3-50172	
CB 1954	5-Aziridinyl-2,4-dinitrobenzamide	
Melphalan	4-[Bis(2-chloroethyl)amino]-L-phenylalanine; p-di(2-chloroethyl)amino-L-phenylalanine; L-phenylalanine mustard; melfalan; L-sarco= lysine; Alkeran; CB 3025; NSC-8806	

Table 1.--Widely used alkylating agents (Continued)

Chlorambucil	4-[Bis(2-chloroethyl)amino]benzenebutanoic acid; 4-[p-[bis(2-chloroethyl)amino]phenyl]= butyric acid; γ-[p-di(2-chloroethyl)amino= phenyl]butyric acid; N,N-di-2-chloroethyl- γ-p-aminophenylbutyric acid; chloraminophene; Leukeran; CB 1348; AI3-26083; NSC-3088	
EMS	Ethyl methanesulfonate; methanesulfonic acid, ethyl ester; AI3-26396; NSC-26805	
HN1	2-Chloro-N-(2-chloroethyl)-N-ethylethanamine; 2,2'-dichlorotriethylamine; bis(2-chloroethyl)= ethylamine; ethylbis(2-chloroethyl)amine; AI3-16200	
HN2	Nitrogen mustard; 2-chloro-N-(2-chloroethyl)-N- methylethanamine; 2,2'-dichloro-N-methyl= diethylamine; N-methyl-2,2'-dichlorodiethyl= amine; di(chloroethyl)methylamine; methylbis(β- chloroethyl)amine; methyldi(2-chloroethyl)amine; chlormethine; mechlorethamine; MBA	
MMS	Methyl methanesulfonate; methanesulfonic acid, methyl ester; AI3-50052; NSC-50256	
Mustard gas	Sulfur mustard; 1,1'-thiobis[2-chloroethane]; bis(2-chlorethyl)sulfide; β,β'-dichloroethyl sulfide; 2,2'-dichlorodiethyl sulfide; bis(β- chloroethyl)sulfide; 1-chloro-2-(β-chloro= ethylthio)ethane	

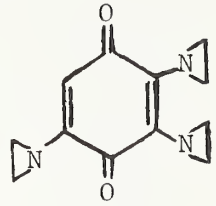
TEM — Tretamine; 2,4,6-tris(1-aziridinyl)-s-triazine; 2,4,6-tris(ethylenimino)-s-triazine; 2,4,6-triethylenimino-1,3,5-triazine; triethylenemelamine; Triamelin; Triethanomelamine; AI3-25296; NSC-9706



tepa — Triethylenephosphoramide; tris(1-aziridinyl)=phosphine oxide; 1,1',1"-phosphinylidynetris=aziridine; phosphoric acid triethylenemide; aphoxide; APO; AI3-24915

Thiotepa — Triethylenethiophosphoramide; tris(1-aziridinyl)phosphine sulfide; 1,1',1"-phosphinothioylidynetriaziridine; Tifosyl; Tespamin; AI3-24916; NSC-6396

Trenimon — 2,3,5-Tris(1-aziridinyl)-p-benzoquinone; 2,3,5-tris(aziridino)-1,4-benzoquinone; 2,3,5-tris(ethyleneimino)benzoquinone; 2,3,5-tris(1-aziridinyl)-2,5-cyclohexa=diene-1,4-dione; Bayer 3231; AI3-51086; NSC-29215



$$R - Y \xrightarrow{\text{slow}} R^+ + Y^-$$

$$R^+ + Y^- \xrightarrow{\text{fast}} RX$$

where Y = leaving group and X = nucleophile.

When the solvated carbonium ion is formed, it reacts rapidly with any nucleophile in the system. While dependent on the rate of ionization, the S_N1 mechanism is largely independent of the concentration of the reactive electron-rich centers. In the bimolecular mechanism, the transfer of the R entity is accomplished through a transition state in which R is loosely combined with both X and Y, and bond formation and bond breakage occur simultaneously.

$$R - Y + X^- \longrightarrow [{}^{-\delta}X \ldots {}^{+\delta}R \ldots {}^{-\delta}Y] \longrightarrow R - X + Y^-$$

Thus the rate of reaction of an agent by an S_N2 mechanism is entirely dependent on the concentration of the nucleophilic centers. Although many biological alkylating agents react by the bimolecular mechanism, some react by a combination of S_N2 and S_N1 and are dependent on conditions of pH and reactant concentration.

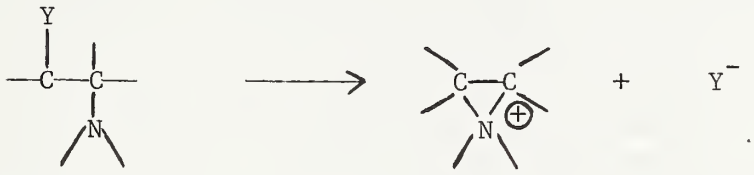
Under physiological conditions, the positively charged entity R^+ will react with any nucleophilic center, e.g., organic and inorganic anions, amino and thiol groups, but there will be essentially no reaction with electrophilic or nonpolar moieties. Thus, theoretically, alkylating agents can react with many cellular components--e.g., nucleic acids, proteins, enzymes, and coenzymes--that possess electron-rich centers.

Biological Effects

In antitumor test data of alkylating agents, the evidence shows that these agents react effectively with rapidly proliferating tissues on three dosage levels. Small doses delay or prevent cell division (cytostatic effect); larger doses induce the formation of cells with altered genetic properties (mutagenic effect); and still larger amounts cause death of the cell (cytotoxic effect). Unfortunately, it is not clear how far the biological effects of alkylating agents depend on cytostatic, mutagenic, or cytotoxic action, although the prevalent opinion is that these effects are related to the alkylating ability of the agent (Krauss 1969; Whitelock 1958). Some workers have suggested that since alkylating agents can exert mutagenicity, then it follows that these may also possess carcinogenicity. Indeed some carcinogenic activity has been associated with alkylating agents, e.g., nitrogen and sulfur mustards (Hueper and Conway 1964), epoxides (Van Duuren et al. 1963, 1965, Van Duuren and Goldschmidt 1966), and alkyl sulfonates (Haddow 1958). With aziridines, however, reports of carcinogenic effects are limited, and there is no proven connection between this carcinogenic activity and the mutagenic activity (Hayes 1964).

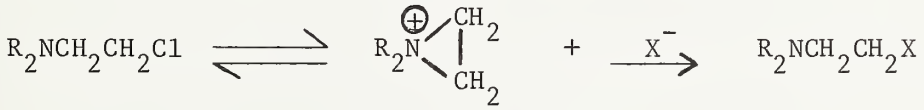
Reported biological effects of alkylating agents have motivated many investigations of carcinostatic as well as carcinogenic effects of these agents. Investigations of these agents have also extended to chemical sterilization of insects (Borkovec 1966, Labrecque and Smith 1968), in which aziridines possess the highest activity. Mustards, in general, are highly toxic when tested as insect chemosterilants at levels similar to those of aziridines. Although these two classes of alkylating agents differ in insect-sterilizing ability, a rational association of aziridines and mustards is reflected in their similar chemical structures.

A general synthetic method of generating an aziridine ring is a unimolecular rearrangement of a vicinally substituted amine to an iminium salt



where Y = leaving group.

This intramolecular alkylation occurs more or less readily, depending on the structure of the substituted amine and the properties of the solvent. Goldberg (1945) suggested that the basic nitrogen mustard acts by a bimolecular mechanism following the formation of a cyclic iminium ion, thus reacting in a manner identical to an activated (protonated) aziridine.



Under neutral or physiological pH, the unimolecular conversion to the iminium ion is relatively fast, and subsequent alkylation of the nucleophilic center proceeds by an S_N2 mechanism dependent on the concentration of the reacting centers. In contrast to aziridines, an acidic medium depresses the alkylating properties of nitrogen mustards, presumably from acid inhibition of aziridinium ion formation.

Gilman and Philips (1946) related the physiological action of mustards to the rate of formation and decomposition of this cyclic iminium intermediate. Suggestions that toxicity of a nitrogen mustard could be correlated to this reactive intermediate led to further investigations (Fruton and Bergmann 1946, Golumbic and Bergmann 1946, Guvin et al. 1947). Interest in such ions has been maintained because of the activity of nitrogen mustards as antitumor drugs (Whitelock 1958, Krauss 1969); their aziridinium forms were studied in connection with antitumor activity. However, antitumor activity has not been unequivocally related to the rate or extent of cyclization to aziridinium ion (Izumi 1955). Such correlation has also been sought in the mutagenic effects of these mustards (Powers et al. 1954), with similar success.

At the cellular level, alkylating agents are most cytotoxic to rapidly proliferating tissues. They may react extensively with resting cells, but cytotoxicity is seen only if these cells are stimulated to divide. Many nuclear degenerative changes, including chromosomal aberration (Auerbach and Robson 1946, Koller 1947) and enlargement of nucleus (Caspersson et al. 1963, Cohen and Studzinski 1967), are observed in vitro and in vivo following treatment with the drugs. Rweckert and Mueller (1960) proposed that cell death, following minimal lethal doses of alkylating agents, is due to unbalanced growth, the various stages of RNA and protein synthesis being out of phase with DNA synthesis. Since alkylating agents react under appropriate conditions with a variety of cellular nucleophilic centers, it is possible that alkylation of any one of a number of sites is the cause of cytotoxicity, but more probably toxicity of minimal lethal doses results from an attack at sensitive sites only. Various mechanisms of action have been proposed (Ross 1962, Wheeler 1962, Warwick 1963) to explain the cytotoxic and mutagenic effects of these chemicals upon rapidly proliferating cells. Quantitative studies have shown that alkylating agents are effective at extremely low intracellular concentrations. Modes of action include inhibition of protein synthesis (Nadkarni et al. 1958, Pradhan and West 1960, 1964), enzymes and coenzymes (Bullock 1955, Kroeger and Holzer 1960), glycolysis (Roitt 1956), and nucleic acid synthesis (Lawley and Brookes 1965). Although all the proposed modes have been investigated experimentally, the investigations have predominantly dealt with the effects upon nucleic acids. Consequently, a hypothesis in which the alkylating agents react directly with the genetic material, deoxyribonucleic acid, thereby destroying or modifying its biological function, has been proposed (Goldacre et al. 1949, Brookes 1964). In this theory three types of damage to DNA can occur, either individually or simultaneously: monoalkylation of bases, esterification of phosphate backbone, and interstrand and intrastrand crosslinking of DNA. Many accept this theory because it can be investigated experimentally in vivo or in vitro, and it correlates observations on a wide range of biological systems.

Base Alkylation

Brookes and Lawley (1960, 1961a, 1961b, 1962) have extensively studied alkylations of DNA by monofunctional alkylating agents; suggested primary sites of attack are shown in figure 1. The main product of base alkylation

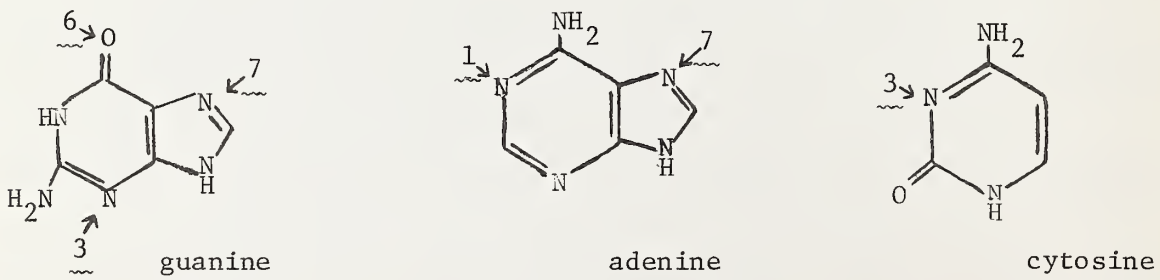


Figure 1.--Akylation sites.

results from a reaction on the N-7 position of guanine. Smaller amounts of other alkylated bases may also be found, e.g., N-3 and N-1 adenine. Lawley et al. (1971-72) have shown alkylation at the N-3 guanine, Loveless (1969) has shown the alkylation at the O-6 position of guanine, and other workers (Ludlum and Wilhelm 1968, Singer and Frankel-Conrat 1969a) have drawn attention to alkylated cytosine. Recently, Singer (1976) has reported that the oxygens in nucleic acid bases react with ethyl nitroso derivatives of urea and guanidine.

In most alkylation studies workers have treated isolated DNA or its components with the more classical alkylating agents, e.g., nitrogen and sulfur mustards and alkyl sulfonates. For example, treatment of DNA with HN2 gave about 70 percent alkylation of the DNA guanine (Doskocil and Sormova 1965a, 1965b). The N-7 atom of guanine was the most reactive site in DNA, yielding mono- and di-guaninyl derivatives, even when large excess of nitrogen mustard was used. Also, a new type of N-7 derivative was described that resulted from reaction of monoguaninyl compound with a second molecule of mustard on the N-atom derived from the first residue of HN2. However, this conclusion, without structural proof, was based specifically on a marked increase in basicity of the derivative without concurrent change of UV spectra. Two additional substituted bases were observed after treatment of DNA with HN2, both bases giving UV spectral evidence that indicated these to be alkylated adenines. In contrast, the reaction of HN2 with the free nucleotide gave two different alkylated adenines; no structural configurations of these alkylated adenines were proposed.

In general, although some structural assignments of products are questionable, evidence supports the theory of N-7 alkylation by nitrogen mustards, sulfur mustards, and sulfate esters. But evidence also shows that the occurrence of various alkylated bases is dependent upon the reaction conditions and isolation techniques (Singer and Frankel-Conrat 1969a, 1969b, Lawley and Thatcher 1970).

In most studies the predominant alkylation of nucleic acid bases occurs at the guanine N-7, but other bases may be attacked at low levels of alkylation, and these may be more significant biologically, in relation to mutagenicity. For example, in vitro reaction of DNA with alkylating agents causes the production of DNA with alkylated sites, with apurinic sites, and with single-strand breaks. In in vivo treatments, endonucleases produce breaks at or near the site of N-7 alkylated bases and subsequent repair is likely. However, these alkylated sites, e.g., O-alkylation, which are not recognized by the endonuclease or are spontaneously lost by depurination, may escape the repair mechanism and become sites at which the mutagenic change has occurred. At higher levels of monoalkylation, breaks are produced immediately in the DNA and its replicating activity is inactivated; a direct result is a cytotoxic effect.

Phosphate Alkylation

In addition to alkylating agents attacking the bases at various positions, some workers have observed esterification of the phosphate backbone of DNA (Alexander et al. 1959, Alexander and Lett 1960, Alexander 1969).

In their early work, these authors used an electrometric titration technique to distinguish between esterification of DNA phosphate and alkylation of base nitrogens. In this technique, esterification by aziridines and epoxides leads to formation of alkali, whereas mustards and sulfate esters generate neither acid nor alkali. Reaction of the base nitrogens with mustard and sulfate esters gives rise to acid, whereas aziridines and epoxides will show no pH change. Under optimum reaction conditions, nitrogen mustards reacted initially with the DNA phosphate groups as measured by this electrometric titration procedure. The evidence indicated that esterification of the phosphate was more important than the alkylation of the purines. However, this titration procedure is only qualitative, and direct quaternization of the tertiary N-7 nitrogen of the DNA purines would have given identical results. Reiner and Zamenhof (1957) showed that N-7-methyl guanine and N-7-methyl adenine were formed after treatment of DNA with dimethyl sulfate, but they did not observe phosphate esterification. However, the absorption spectra and chromatographic behavior of alkylated derivatives from the treatment of DNA with HN1 showed mainly phosphate attack, while diethyl sulfate gave only primary phosphate esterification.

Alexander and Stacey (1959) confirmed that MMS produced N-7 purines, while EMS presumably combined with the phosphate backbone of DNA. With an aromatic nitrogen mustard (CB 3025), Alexander and Lett (1960) also observed an initial formation (> 90 percent) of phosphate esters. With some alkylating agents, initial esterification of the phosphate group occurred under physiological conditions; then the phosphate ester group interacted with an aromatic tertiary amine to give a quaternary nitrogen product (transalkylation). Andrews et al. (1952) first observed intramolecular transalkylation in their chemical studies. Under mild conditions the methyl group of a phosphate triester quaternized a pyridine residue; the ethyl phosphate ester alkylated at a much slower rate. Using these data, Alexander and Stacey (1958) proposed a similar reaction with deoxyribonucleic acid to explain their observations.

Evidence for transalkylation at physiological conditions was obtained for monofunctional alkylating agents by definite changes in molecular weight, size, and ultraviolet absorption of DNA at various periods after the initial alkylation. Measurements made immediately after the initial reaction showed the DNA to have a coiled structure (i.e., its size contracted to a smaller solution volume) without a decrease in molecular weight; this was attributed directly to esterification of phosphate groups. After the reaction of the monofunctional nitrogen mustard [$(CH_3)_2NCH_2CH_2Cl$] (Alexander et al. 1961, Lett et al. 1962) with DNA was complete, the absorption spectrum of the DNA was unaltered, but, on standing, a new chromophore at 280 nm, characteristic of N-7 alkylated guanine (Brookes and Lawley 1961b), slowly appeared. With this mustard, the main sequence of reactions appeared to be: (1) esterification of the phosphate group, (2) transfer of some of the alkyl ester groups to form a quaternary base, (3) elimination of the base after hydrolysis of sugar-base link, and (4) hydrolysis of the phosphate diester bond (main chain scission) after elimination of purine. Thus a slow decrease in molecular weight of alkylated DNA was observed and attributed to breakage of the DNA phosphate backbone. Experiments with native DNA showed that alkylation caused the release of 7-ethylguanine (Bautz and Freese 1960, Brookes and Lawley 1961a) and of some

3-alkyladenine (Lawley and Brookes 1963). The depurinated DNA strand can break by β-elimination, similar to depurination of DNA by acid hydrolysis (Tamm et al. 1953). In fact, Lawley and Wallick (1957) determined that 7-methylguanine was slowly liberated at neutral pH after alkylation.

Bannon and Verly (1972) showed that phosphate alkylation represented 15 percent of total alkylation when DNA was alkylated with EMS and only 1 percent of total with MMS. They also showed that the phosphate triesters from alkylation of DNA by EMS were very stable: most remained intact when heated at 100 °C for 90 minutes at pH 7.0. These results (with EMS) contradict the opinion of Alexander and Stacey (1958) that the conditions and high temperatures used to hydrolyze DNA favor transalkylation. The low alkylation level with MMS was explained in that the process of transalkylation from phosphate to base occurred in DNA with the methyl group and not the ethyl group. At the same alkylation dose, phosphate alkylation in DNA is much more important with EMS than with MMS; phosphate alkylation in DNA with MMS is almost negligible. This may reflect a difference in the alkylation mechanism: MMS has been shown to react exclusively by a S_N2 mechanism, while EMS has been shown to react by a combination of the two mechanisms, S_N1 and S_N2 (Ross 1962).

Although phosphate alkylation had marginal support, the formation of phosphate esters in DNA treated with alkylating agents proved difficult to measure and subsequently was abandoned by some supporters as a more important reaction than N-7 base alkylation. Nevertheless, Rhaese and Freese (1969) showed that thymine did not react with MMS or EMS, but deoxythymidylic acid was alkylated exclusively at the phosphate group, about twice as fast as guanine at the 7-position. The alkylation of oligodeoxythymidylic acids produced products, separated by chromatography, which proved the existence of triester breakage. Thus, with some alkylating agents, phosphate alkylation may indeed be important in the biological activities of these compounds.

An overview of DNA phosphate alkylation might involve the following theoretical consideration: esterification of DNA can lead to mutations, by producing no more than a minor alteration or miscoding in the DNA so that its biological replication is not prevented, but only slightly hindered. It is this altered DNA that represents the mutant. According to this view, the alkylation of the DNA does not produce a mutation but merely increases the chance that a mutation will be produced in the subsequent synthetic processes. If the mutagenic agent modifies the DNA too severely (e.g., cross-links it), it will be nonreplicating and new mutated DNA will not be produced.

Crosslinking

Difunctional alkylating agents exert a cytotoxic effect when applied at selected doses. Alkylating agents supposedly prevent replication of the macromolecule DNA with the formation of interstrand or intrastrand crosslinks, and subsequent chromosomal damage by strand breakage (Biesele et al. 1950). Such crosslinks arise from an attack on neighboring guanines since derivatives consisting of two guanines, joined by the residue of an alkylating agent, have been isolated from DNA hydrolyzates (Lawley and Brookes 1963).

Physical evidence for the existence of interstrand crosslinks was obtained by reversible denaturation experiments (Geiduschek 1961, 1962, Becker et al. 1964). When DNA is heated in solution to a temperature sufficient to separate the twin strands, there is a consequent increase in UV absorption. If the process is rapidly reversed, crosslinked DNA will almost return to the original ordered structure. With intrastrand crosslinked or monofunctionally alkylated DNA, heat denaturation and rapid cooling does not allow a reformation of the original DNA helix. Further evidence is obtained by density-gradient centrifugation in cesium chloride. Samples of denatured, nonalkylated or monofunctionally alkylated DNA are banded in high molarity cesium chloride and show an increase in density associated with denaturation. However, crosslinked DNA is banded at a density corresponding to the original native DNA.

This crosslinking theory works well for in vitro studies with isolated DNA and some alkylating agents but fails in most in vivo studies. For example, Brewer and Aronow (1963) reported the treatment of DNA, isolated from mouse fibroblasts growing in vitro, with a low, effective dose of HN2. Comparison of physical properties of treated and untreated DNA isolated from culture substrates for pancreatic DNase indicated that there was no difference between the two preparations of DNA. The only noticeable difference was that the HN2-treated DNA was slightly more viscous than normal DNA. In contrast to these findings, DNA which had been allowed to react in vitro with high concentrations of HN2 showed marked differences from untreated DNA based upon reversible denaturation and density gradient centrifugation. The authors concluded that the data did not support the hypothesis that crosslinking of DNA was responsible for the growth-inhibiting property of HN2.

INTERACTION OF ALKYLATING AGENTS WITH OTHER CELLULAR COMPONENTS

It is possible that the biological effectiveness of alkylating agents has no quantitative relationship to the extent of alkylation and that the specific type of interaction is more important than the total extent of alkylation. Nucleic acid synthesis, particularly DNA synthesis, is readily inhibited in many cell systems after exposure to small amounts of alkylating agents (Lawley and Brookes 1965, Levis et al. 1963). Interference with nucleic acid synthesis can occur at any time in the cell cycle (Wheeler and Alexander 1964a, 1964b, 1969, Goldstein and Rutman 1964, Venitt 1971), causing inhibition with the functions of the cell, e.g., (a) synthesis of ribonucleotides, (b) conversion of purine ribonucleotides into components of DNA, (c) deoxyribonucleotides incorporating enzymes, and (d) primer activity of nuclear material.

With purified DNA templates and polymerase enzymes, the effects of alkylating agents on nucleic acid synthesis have been investigated to completely define their mechanism of action at the molecular level. Ruddon and Johnson (1968) found that treatment of calf thymus DNA with low concentrations of HN2 (5×10^{-7}M) produced a significant inhibition of DNA template activity in a purified Escherichia coli RNA polymerase system; it took concentrations of HN2 20 to 100 times higher to achieve an equivalent amount of inhibition of DNA synthesis utilizing the DNA polymerase system. The template activities of both native and denatured DNA were inhibited by HN2. The percent of inhibition of DNA template activity was correlated with the

number of alkylations per 10^4 nucleotide units by measuring the binding of ^{14}C-HN2. Ruddon and Johnson's data also showed that the template function of DNA as it exists in the nucleoprotein complex was insensitive to the HN2 treatment. This suggests that DNA may not be the most sensitive site to alkylation and that other sites of action may be more important for the cytotoxic effect of HN2.

Riches and Harrap (1973) reported that the biosynthesis of histones was inhibited from the outset by a nitrogen mustard, chlorambucil, while non-histone protein biosynthesis declined 12 hours after treatment. These results indicated that the mustard could bring about extensive change in the chromatin structure and might be a necessary prerequisite to the changes in DNA synthesis and, consequently, to cell death. Indeed this could also explain the low levels of an alkylating agent needed for biological activity.

INTERACTION OF AZIRIDINES WITH DNA

The biological activities of aziridines have greatly contributed to the continued interest in their physiological fate over the past 30 years, as many investigators have attempted to find their primary mode of action. A large portion of these studies have attempted to gather evidence supporting the hypothesis of a direct interaction between the aziridine and the genetic material. The evidence can be divided into three distinct areas: base alkylation, esterification of phosphate backbone, and crosslinking of DNA.

Base Alkylation

Prompted by kinetic studies of the mutagenic effect of TEM (Iyer and Szybalski 1958), Lorkiewicz and Szybalski (1961) conducted two types of experiments and showed that TEM interacted primarily with phosphorylated DNA pyrimidines and not with DNA purines. The first was a chromatographic analysis to distinguish products from DNA precursors. Low concentrations of TEM in aqueous solution, in combination with base, nucleoside, or deoxynucleotide, were held at low temperatures (4 °C) for 6 days. Chromatography of the reaction mixtures did not reveal any interaction with the DNA purines or their corresponding ribosides and deoxyribosides. In contrast to purines, both deoxycytidine and deoxythymidine and their ribosides apparently interacted with TEM. Elution of a spot with the same R_1 value as deoxycytidine from a paper chromatogram and evaluation by UV spectroscopy indicated a material different from deoxycytidine. Also, the UV spectrum of this eluted spot could not be obtained by composite spectrum of deoxycytidine and TEM. Elution and UV analysis of the deoxythymidine and TEM spot revealed only subtle differences when compared to the deoxythymidine spectrum; the product, if any, was not identified.

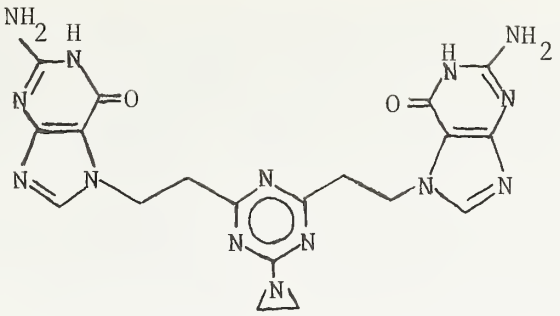
In the second phase of the experiment, the mutagenic activity of the reaction mixture was compared with activity of separate entities, i.e., TEM, base, and deoxyribosides. Assessment of the mutagenic activity of the reaction products revealed that the interaction of TEM and deoxycytidine led to almost complete loss of mutagenic activity against *Escherichia coli* Sd4 strain, whereas the mutagenicity more than doubled when TEM reacted with deoxythymidine. In contrast to pyrimidines, neither TEM alone nor in combination

with the purines increased or decreased the mutagenic activity against the Sd4 strain. Lorkiewicz and Szybalski (1961) did not propose structures for the products, but they did attempt to isolate these materials by chromatography with this mutagenic activity as the method of detection. However, the mutagenic product was lost, possible destroyed by the chromatographic system employed.

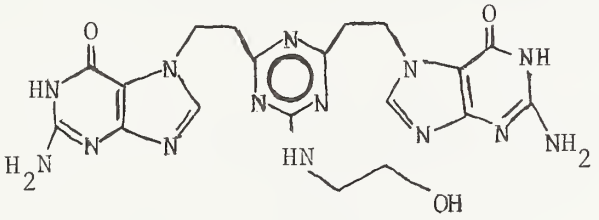
Results of this work suggest that TEM does not react with the purine precursors of DNA but that it does react with one or both of the pyrimidine precursors, derivatives of cytosine and thymine, though the chemistry of these interactions has not been clarified. In any case, this paper does not present any evidence for or against the direct interaction of TEM with already formed DNA.

Doskocil (1965) conducted a study on base alkylation of DNA by TEM only to contradict the observed interaction of TEM with the DNA pyrimidines (Lorkiewicz and Szybalski 1961). When Escherichia coli-DNA labeled with ^{14}C purines was allowed to react in neutral aqueous buffers at 37 ^{o}C for 6 hours, hydrolyzed by concentrated formic acid, and then chromatographed on paper, a new UV-absorbing spot was detected. In vivo treatment of the same bacteria with TEM followed by isolation of DNA, hydrolysis, and chromatography showed a small but detectable amount of radioactivity in the zone of the new compound. Since only the purines of the DNA were labeled, this result implied that TEM reacted with the purines and not with the pyrimidines. Doskocil suggested that this spot was a diguaninyl derivative after he compared the chromatographic and spectroscopic properties of the guanine N-7 derivative obtained from a reaction of DNA with HN2 (Brookes and Lawley 1961a) and of the product from a reaction of DNA with TEM. The very small amount of substituted guanine found indicated a low level of alkylation by TEM, also evidenced by less than 2 percent reaction with total guanine present. Although the structure of the presumed guanine derivative was not identified, this paper presented the first evidence of a direct interaction between an aziridine and DNA. However, the extent of this reaction was very small (less than 2 percent). Under similar experimental conditions with nitrogen mustards (Doskocil and Sormova 1965a, 1965b), about 70 percent of DNA guanine reacted.

Lawley and Brookes (1967) presented further evidence for base alkylation; TEM was shown to react with DNA and DNA purines, again in contrast to claims by Lorkiewicz and Szybalski (1961). The four deoxynucleosides were treated with TEM in a phosphate buffer at 4 ^{o}C, and analysis by paper chromatography showed the presence of two new spots, but only with deoxyguanosine. Detection of one of these blue fluorescent spots under short wave UV irradiation occurred only after 72 hours; the second spot became apparent only after 170 hours. The reaction of TEM with deoxyguanosine monophosphate and with salmon sperm DNA at 37 ^{o}C and pH 7 yielded the same two bases after mild acid hydrolysis of the reaction mixtures. Isolation of these materials from ion exchange resins failed even when the column was eluted with dilute HCl; the authors stated that TEM reacted with the column material. They also suggested that one of these materials was a composite of one molecule of TEM and two molecules of guanine based upon the ultraviolet analysis of the major product eluted from paper.



However, with this structure Lawley and Brookes have assumed that one aziridinyl function would remain, which would be unlikely after acid hydrolysis of alkylated DNA or nucleotide. The acid hydrolysis of the reaction mixture would destroy the remaining reactive aziridine functions (Beroza and Borkovec 1964). A more reasonable structure would be the following:



Even though the structural assignment is questionable, the authors present data to strengthen the hypothesis of a direct interaction between TEM and the guanine portion of DNA. It is not clear, however, whether the product obtained by Lawley and Brookes (1967) was the same as that described by Doskocil (1965). Also Lawley and Brookes (1967) did not clearly express the extent of reaction of TEM with DNA in terms of percent guanine.

Other workers have investigated various aspects of the reaction of aziridines with nucleic acids or their bases. Mellett and Woods (1961) reported an interaction of thiotepa with guanosine and dGMP, but they did not include experimental conditions and evidence in their report. Hieble et al. (1973) presented NMR evidence for the reaction of N-acylated ethylenimines with 9-alkyl guanine in acetic acid to form N-7 derivatives, but there is no precedence for such an analogous reaction under neutral aqueous conditions.

Lashkova et al. (1973) observed a chemiluminescence in the reactions of nucleic acid components, e.g., ammonium deoxyadenine 5-monophosphate, sodium guanosine 5-monophosphate, deoxyguanosine, and 2-deoxy-D-ribose, with N-acetylethylenimine. No luminescence was observed when N-acetylethylenimine was added to solutions of the DNA bases. Also, the luminescence was greater with purine nucleotides than with pyrimidine nucleotides. The authors

suggested that this chemiluminescence may result from oxidation of the glycosidic carbon atom of the DNA carbohydrate moiety, but no evidence was presented. While Lawley and Brookes (1967) did not observe chemiluminescence when TEM was allowed to react with deoxyguanosine and deoxyguanosine monophosphate, they did observe fluorescent products under UV light and they rationalized that these were N-7 alkylated purines. Although Lawley and Brookes (1967) presented no evidence to support an interaction of TEM with the carbohydrate moiety of the DNA components, the vast differences in chemical reactivities of TEM and N-acetyethylenimine could explain these different observations.

Tomasz (1970a, 1970b) developed a simple and selective method to measure the extent of 7-alkylation of guanine residues in alkylated nucleic acids. Methylation of the N-7 position of guanine derivatives renders the C-8 hydrogen extremely labile, i.e., C-8 position of 7-methylguanosine rapidly exchanges hydrogen with the solvent. Consequently, a measure of released tritium from the C-8 position gives the extent of N-7 alkylation. The exchange mechanism is a simple acidic dissociation of the C-8 proton, and not tautomerism or reversible hydrolytic opening of the imidazole ring. When (8-^{3}H)guanosine and (8-^{3}H)guanine-DNA were methylated with dimethyl sulfate and the amount of released tritium from the DNA was measured as tritiated water, the results corresponded to the amount of 7-methylguanine. Release of tritium from DNA labeled at the 8-position of guanine was measured after interaction of DNA with the bifunctional nitrogen mustard HN2, the trifunctional aziridine TEM, and the antibiotic mitomycin C. The nitrogen mustard showed a direct correlation between amount of guanine alkylated (measured by determining the decrease of total guanine) and the amount of tritium released by exchange. Treatment of (8-^{3}H)guanosine in phosphate buffer with HN2 for 1 hour at neutral pH yielded 29 percent alkylation, while alkylation of (8-^{3}H)guanine-DNA showed more than 50 percent alkylation.

Under comparable conditions, incubation of (8-^{3}H)guanosine with TEM for 7 hours at ambient temperature resulted in a 49 percent release of tritium from C-8 position; incubation of (8-^{3}H)guanine-DNA with TEM caused substantial release of tritium in 7 hours (54 percent) and in 18 hours (58 percent). The percent alkylation for HN2 is comparable to that observed by other workers, but percent alkylation by TEM is much greater than the low level (2 percent) measured by Doskocil (1965).

The difference may have been caused by the large molar excess of TEM used in the tritium assay. When mitomycin C was used under optimum in vitro conditions for covelent binding of the drug to DNA, no exchanged tritium was detected. Thus mitomycin C, which possesses an aziridinyl moiety, did not alkylate the N-7 position of the guanine residues in DNA as proposed by many workers. However, as this assay procedure indicates only alkylation at guanine N-7, it is possible that other positions in the macromolecule were alkylated. Such alternate alkylations are supported by recent findings of O-alkylation by other alkylating agents, e.g., O-6 alkylation of guanine by ethylnitrosourea (Singer 1976).

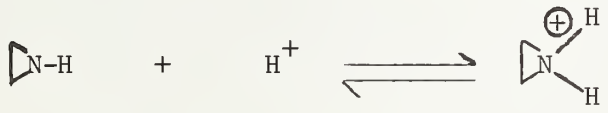
Thus Doskocil (1965) and Lawley and Brookes (1967) have presented evidence that TEM appears to react with DNA guanine at the N-7 position. In

contrast, however, Lorkiewicz and Szybalski (1961) have shown TEM to interact only with the pyrimidines. There is also substantial disagreement on the extent of reaction: Doskocil (1965) reported less than 2 percent reaction of DNA guanine with TEM, while Tomasz (1970b) reported greater than 50 percent reaction of TEM with DNA guanine; in all cases, the structure (or structures) of the product (or products) has not been unequivocally identified. Unlike reports of isolation and structural identification of products derived from other alkylating agents, similar analyses for aziridines have evaded many workers, with only UV spectra and paper chromatographs offered as conclusive proof of base alkylation. Also, in these studies only TEM has been used; there is no evidence that other aziridines react in the same way.

Phosphate Alkylation

Unlike the studies of DNA phosphate alkylation by nitrogen mustards and sulfate esters, reports involving the phosphate esterification by aziridines are only marginal. For example, Alexander et al. (1959, 1961) studied the reactivity of a difunctional aziridine (CB 1263) with sperm heads by gel pellet formation only to demonstrate that CB 1263 was more effective than aromatic nitrogen mustards, but the low reaction level was beyond the sensitivity of the electrometric titration technique which was used to detect phosphate alkylation.

Electron microscopic analysis of DNA treated with ethylenimine showed uniform staining along the length of the molecule, which suggested that the ethylenimine reacted with DNA at equally spaced positions, probably at the phosphate linkages (Ulanov and Kruglyakova 1967). Pomonis et al. (1971) proposed a mechanism in which the aziridine moiety is attacked by a nucleophilic HPO_4^{-2} ion. If a steady concentration for the aziridinium ion is


(1)


(2)


(3)

assumed (equation 1), then the nucleophilic HPO_4^{-2} ion will attack the

protonated aziridine (equation 2) in competition with water (equation 3). At a neutral pH of a concentrated phosphate buffer, the high concentration of the HPO_4^{-2} ion will attack the aziridinium ion, acting as a stronger nucleophile than water. This could be the case with the phosphate DNA backbone.

Although there is no direct evidence for alkylation of phosphate groups by aziridines in vitro or in vivo, it cannot be entirely excluded that alkylation of the bases takes place in some cases by initial phosphate esterification followed by transalkylation. Chmielewicz et al. (1967) and Bardos et al. (1965a) have speculated that a nucleophilic attack on the aziridine rings by the phosphate functions of the DNA backbone occurred, followed by a rapid transalkylation to the guanine moiety. The authors have observed a lowering of the T_m after treatment of DNA with various aziridines. Continued incubation of the DNA with the aziridines caused a marked change in the UV spectrum of the nucleic acid similar to that reported by Alexander et al. (1961), using a monofunctional nitrogen mustard.

Singer (1976) has shown that hydrolysis by enzymatic methods is required to investigate the products of DNA and alkylating agents since all O-alkyl pyrimidines are labile in acid and alkali, so that losses (up to 100 percent) occur during the degradation of an alkylated nucleic acid unless neutral enzymatic digestion is used. Furthermore, separation of the products in a digest must be done under conditions which preserve the O-alkyl linkages. This could also apply to possible phosphate esters formed by aziridines.

Crosslinking

The predominant approach to examining effects of difunctional alkylating agents on DNA involves a measurement of crosslinks formed after exposure of macromolecules to alkylating agents. A variety of methods is used, i.e., reversible denaturation, cesium chloride gradient, gel pellet formation, and isolation of crosslinked product. Investigations of crosslinking by mustards supported by many physical data are numerous (Kohn et al. 1965, 1966, Kohn and Spears 1967, Walker 1971), but the supporting data for crosslinking by aziridines are less convincing.

An early method used by Alexander et al. (1959) involved the in vitro reactivity of several classes of biologically active alkylating agents with nucleoproteins from herring sperm heads (consisting of one-third DNA and two-thirds protamines). Treatment of a suspension of sperm heads in dilute saline with a bifunctional agent resulted in crosslinking of DNA, which could be isolated as a gel. The protein did not take part in this crosslinking reaction, which was confined to the joining together of different DNA molecules. Treatment of the sperm heads with a difunctional aziridine (CB 1263) resulted in sedimentation of 90 percent of the DNA as a gel pellet indicative of crosslinks. Even at this level the protamines were not involved in the crosslinking as all protamines were in the supernatant. On this basis this difunctional aziridine was a better crosslinking agent than aromatic nitrogen mustards, e.g., chlorambucil. But estimation of the percent alkylation by an electrometric titration method was unsatisfactory, since the low level of alkylation was beyond the sensitivity of the method.

Doskocil (1965) reported that TEM reacted with the purines of bacterial DNA labeled with ^{14}C-purine. A substituted base in the hydrolyzate was isolated and identified spectrophotometrically as a guanine substituted with TEM at the N-7 position, in a molar ratio of guanine:TEM 2:1. The DNA treated with TEM was apparently crosslinked, since no permanent strand separation could be achieved by thermal denaturation. Although the thermal denaturation of DNA from TEM-treated cells showed some degree of reversibility, the nearly complete reversibility shown by DNA from in vitro treatment with TEM was usually not observed. After thermal denaturation, the DNA treated in vivo showed neither permanent hyperchromicity nor perfect reversibility; thus only a part of DNA molecules had interstrand crosslinks. In resting bacteria, TEM produced interstrand crosslinking of DNA and also crosslinking of DNA with protein, while DNA isolated from cells treated in vivo was degraded and partially denatured. The maximum hyperchromicity was lower in DNA from TEM-treated cells than in DNA from control cells. This indicated a partial denaturation; no decrease of the maximum hyperchromicity was observed in DNA treated with TEM in vitro. The lowering of the hyperchromic effect was even more pronounced in "inextractable DNA" that was liberated by means of trypsin and chymotrypsin.

Doskocil noted that TEM was much less reactive than HN2; under the conditions used in the experiments, less than 2 percent of guanines were substituted with TEM, whereas 70 percent of DNA-guanine would have reacted with nitrogen mustards under comparable conditions (Doskocil and Sormova 1965a, 1965b). This could be explained by subsequent breakdown of DNA, liberating free noncomplementary fragments from reversible DNA initially present. If the formation of crosslinks is rapid in comparison with DNA breakdown, reversible DNA may easily be isolated. If the crosslinks are formed at the speed comparable to the velocity of the breakdown, the isolated DNA would show only incomplete reversibility. Doskocil (1965) stated that this could be the case with TEM.

This study (cited earlier) postulates that DNA-guanine interacts with TEM and that this reaction leads, at least initially, to crosslinking of the DNA. Presumably, at least two of the aziridinyl groups of the TEM molecule react with two guanine moieties located in two different strands of the DNA molecule. This work also suggests that crosslinking is a sensitive tool for detecting direct alkylation of DNA when the extent of the reaction is very low.

Lawley and Brookes (1967) produced experimental data showing that in vitro, TEM-treated DNA had properties characteristic of crosslinked DNA. The ability for renaturation increased as the extent of reaction with TEM increased and could be detected at approximately 1.5 mmole TEM/mole DNA-phosphorus. Treatment of DNA, which had been isolated from various sources, revealed differences in the amount of alkylating agent required to produce renaturation. This is explained by differences in guanine and cytosine content. The authors stated that measurement, if possible, of the proportion of crosslinked guanine products in DNA by chemical analysis would be the best estimation for interstrand crosslinks since in vivo experiments yield very low levels of alkylation. But, as shown later (Lawley et al. 1969, Flamm et al. 1970), alkylating agents reacted with DNA and formed intrastrand crosslinks; therefore direct chemical analysis would overestimate interstrand

crosslinks. Furthermore, these intrastrand crosslinks appeared to represent a significant factor in the production of cellular toxicity and cannot be ignored in DNA alkylation studies (Flamm et al. 1970). In general, these reports support the conclusion of Doskocil (1965) in that TEM reacts with DNA-guanine and that this reaction is accompanied by crosslinking. Even though the possible differences between interstrand and intrastrand crosslinks are discussed, no new evidence is presented about the alkylating process and the structure of its products.

In studying the crosslinking theory, Chmielewicz et al. (1967) incubated (37 ^{o}C) solutions of calf thymus DNA with several bis(1-aziridinyl)phosphinyl urethanes and HN2 at various concentrations, pH, and incubation periods. These compounds lowered the helix coil transition temperature of the DNA if the incubations were conducted at pH 5.3. But at pH 7.0, only HN2 showed a significant effect; nevertheless, even this compound was more effective at slightly acidic pH. This unexpected result was explained by the apparent "loosening" of the double helix of DNA at pH 5.3, as indicated by the lower T_m of the control; this would result in greater exposure of the base nitrogens to alkylation. Also, the authors observed a further lowering of the T_m upon continued incubation with HN2 or certain aziridines, e.g., AB-132, which are known to hydrolyze completely (Bardos et al. 1965a) during the first few hours and cease to alkylate thereafter. Incubation of DNA with alkylating agents caused a marked change in the UV spectrum of the nucleic acid; Chmielewicz et al. (1967) designated this behavior as a hyperchromic dispersion: the increase of absorbance as a function of wavelength. This phenomenon was believed to be related to the quaternization of the guanine N-7 position. The data showed that the initial hyperchromicity at 276 μm caused by AB-100 was equal to, or greater than, that caused by equal concentration of HN2. Since AB-100 is about 200 times less reactive than HN2 in alkylating nitrobenzyl-pyridine as a model nucleophile (Bardos et al. 1965b), its relatively large initial alkylation was surprising. However, nucleophilic attack on the aziridine rings by the phosphate functions of DNA, followed by a rapid transalkylation to the guanine moiety, could explain this observation. Further reaction of the aziridine, at a higher concentration, would lead to extensive esterification of the phosphate backbone, thus causing scission of the polymer chain. Evidence for breakage of DNA was suggested by the viscosity measurement of the DNA solution treated with the aziridine for different periods. A decrease of viscosity may be attributed to either denaturation of the DNA or to esterification of the phosphate groups. Esterification may also lead to scission due to the instability of the phosphotriester bonds. Under acidic conditions, AB-100 and HN2 appeared equally effective for esterification, but the aziridine was much less reactive than HN2 in causing denaturation. Thus the viscosity-lowering effect of AB-100 was explained by a greater reactivity of the aziridine rings toward the phosphate groups causing esterification and subsequent decrease of chain lengths.

This study is important in that it employed aziridines other than TEM. It proposed a sequence of reactions initiated by the attack of an aziridine group on the DNA-phosphate backbone (esterification), followed by trans-alkylation in which the DNA-phosphate ester functioned as an alkylating agent and the DNA-guanine was the receptor. However, since no products were isolated and chemically identified, all evidence for the proposed mechanism

was derived from the observable changes in the physical properties of treated DNA. In addition to crosslinking, the treated DNA was assumed to undergo some fragmentation, a process which did not occur or was not considered significant by Doskocil (1965) and Lawley and Brookes (1967) in their work with TEM.

Recently, a fluorometric method for nucleic acid estimation (LePrecq and Paoletti 1966) was applied to measuring covalent crosslinking in DNA (Burnotte and Verly 1972). When ethidium bromide binds to DNA by intercalating its duplex region, the weak fluorescence of the dye increases (LePrecq and Paoletti 1964). Crosslinking of DNA is determined by comparing the fluorescence before and after a heat-denaturation step at alkaline pH. Under these conditions, separable strands do not reanneal, and only crosslinked sequences can anneal to increase the fluorescence. When there are no strand breaks, the method is as accurate as the cesium chloride density gradient centrifugation (Iyer and Szybalski 1963). In combination with either isopycnic ultracentrifugation or a S_1-endonuclease assay (Lown et al. 1976), the method can be used to investigate covalent crosslinks induced by alkylating agents.

Lown et al. (1976) studied three aspects of mitomycin C on DNA using this ethidium fluorescence assay: crosslinking, monoalkylation, and strand breakage. At various concentrations of the drug crosslinking of DNA was measured; at relatively high concentrations of mitomycin C before the initial heat denaturation step at alkaline pH, a suppression of the fluorescent was observed. Treatment of a radiolabeled synthetic polynucleotide revealed that no depurination occurred but rather that this suppression was due to monoalkylation of potential intercalation sites. The authors concluded: (1) that the proportional decrease in fluorescence with pH suggested that the alkylation was due to the aziridine moiety of the drug, and (2) the parallel increase in the rate and overall efficiency of crosslinking of DNA with lower pH suggested that the crosslinking occurred sequentially with alkylation by aziridine and then by carbamate. Crosslinking by mitomycin C was also independently confirmed by a S_1-endonuclease assay. No precise binding sites on the DNA were determined, but the authors mentioned a possible reaction at the O-6 position of guanine.

With this fluorometric method, Akhtar et al. (1975) examined a series of aziridinequinones for possible crosslinking of DNA. The aziridines crosslinked DNA efficiently, but with a noticeable dependence on the pH. With the detection of crosslinks, a suppression of the before heat denaturation fluorescent was observed, indicative of monoalkylation. Treatment of a radiolabeled synthetic polynucleotide revealed that no depurination accompanied alkylation of covalent crosslinking. Thus, this evidence suggested that monoalkylation of DNA occurred and destroyed potential ethidium intercalation sites. The treatment of DNA with different guanine and cytosine (G&C) contents, but of comparable molecular weights, showed that a direct relationship existed between maximum crosslinking with higher G&C content for a given concentration of aziridine. When the average number of crosslinks per molecule was estimated, it was comparable with similar estimates made by Iyer and Szybalski (1963) for mitomycin C crosslinking. Although the work by Tomasz (1970b) places serious doubt on the direct alkylation of the N-7 position of guanine by mitomycin C, Akhtar et al. (1975) stated that a possible reaction occurred at the O-6 position of guanine by the structurally

similar aziridinequinones. A kinetic study involving the measurement of rate constants for acid-assisted ring opening of aziridinequinones showed that the pH dependence of the ring opening paralleled the rate of covalent crosslinking and of monoalkylation of DNA. Akhtar et al. (1975) suggested that this pH dependence of crosslinking implied that the active species involved directly in covalent bonding to DNA was the intermediate aziridinium ion. Likewise, Bardos et al. (1965a) have shown that the antitumor activities of TEM, tepa, and AB-132 paralleled their rates of hydrolysis rather than the rates at which they alkylated nitrobenzylpyridine.

In general, these reports support the conclusions of many authors that crosslinking of DNA occurs with aziridines, although the crosslinking is usually pH dependent and at a low level. Common methods to estimate crosslinking by alkylating agents include cesium chloride density gradient centrifugation, hyperchromicity of crosslinked denatured DNA, and more recently, ethidium fluorescence assay. This fluorescent technique appears to be a rapid and dependable method for measurement of DNA crosslinks by alkylating agents. In addition, the measurement of the mean number of crosslinks per DNA molecule does not require precise knowledge of the DNA concentration nor extensive purification of the DNA preparation (LaPrecq and Paoletti 1966). In all cases, however, crosslinking sites for aziridines have not been unequivocally identified. Doskocil (1965) and Lawley and Brookes (1967) have presented weak evidence that crosslinking occurred with TEM, but the expected diguaninyl derivative was not isolated and identified.

Alkylating agents interact with DNA, but an apparent discrepancy exists between in vitro and in vivo observations. In general, to affect the secondary structure of DNA requires from 10 to 100 times higher concentrations of the agent, compared with the estimated in vivo concentrations at biologically effective dose. For instance, damage to DNA by alkylation could not be detected in cells exposed to low, but lethal, concentrations of alkylating agents (Lorkiewicz and Szybalski 1961, Trams et al. 1961b, Brewer and Aronaw 1963). Trams et al. (1961a) measured the incorporation of (^{14}C)-TEM into the DNA fraction of regenerating liver and of various mouse ascite lymphomas. The in vivo incorporation of ^{14}C-TEM was estimated at 1 mole of drug per mole of DNA polymer (or approximately 1 molecule of TEM per 10^5 nucleotides), which suggested that attack of DNA itself did not necessarily represent the primary mechanism of action; in fact the effect of TEM was more pronounced on protein synthesis than on DNA synthesis. However, the fact that DNA alkylation cannot be demonstrated does not imply that the reaction with DNA in vivo is not an important cytotoxic event. The actual amount of alkylating agent covalently bound to DNA represents only a small quantity of the total bound material and the present physical techniques may not be sufficiently sensitive to detect extremely low alkylation levels. No good correlation has even been established between the binding of an agent to cellular DNA and the sensitivity of that cell to alkylation.

McCann et al. (1971) found drug-induced crosslinks in the DNA of chick embryos within 6 hours after injection of mitomycin C and HN2 into the egg and 24 hours after injection of thiotepa. Crosslinked DNA was estimated by a dextran:polyethylene glycol system (Alberts 1967), which separated the crosslinked DNA from denatured DNA after heat denaturation. Effects on the rates

of synthesis of DNA, RNA, and protein were studied with chemical assays for total content of these macromolecules as well as incorporation of radioactive precursor. All three drugs inhibited DNA synthesis before RNA and protein synthesis were affected, but there were discrepancies between the two methods of measuring the DNA synthesis. When the effects of HN2 were compared to those of its monofunctional analog, dimethyl-2-chloroethylamine, the former was the more effective inhibitor of DNA and RNA synthesis at equivalent alkylating doses. However, the fact that the monofunctional analog had some inhibitory activity suggested that not all the effects of difunctional alkylating agents represent crosslinking activity. Venitt (1971), who studied the cytotoxic effects of 5-aziridinyl-2,4-dinitrobenzamide (CB 1954), also concluded that its cytotoxic action at a low reaction level with DNA is not totally represented by simple monoalkylation of DNA. Connors and Melzack (1971) suggested that CB 1954 behaves as an alkylating antimetabolite, blocking purine biosynthesis by competing for and reacting with an enzyme (or enzymes) in the pathway from 4-aminoimidazole-5-carboxamide ribonucleotide to inosine monophosphate; however, Venitt (1971) could find no evidence to support this hypothesis.

INTERACTION OF AZIRIDINES WITH OTHER CELLULAR COMPONENTS

Similar to that shown for other alkylating agents, the biological effectiveness of aziridines may involve a specific type of interaction which is more important than the total level of alkylation.

Chmielewicz et al. (1967) have reported that the template function of calf thymus DNA for RNA synthesis was more sensitive to alkylation with HN2 than its template function in DNA synthesis. Similar treatment of calf thymus DNA with AB-100 caused a significant decrease in its ability to serve as a template. The concentration was of the same order of magnitude as the estimated in vivo concentration of this agent after administration of a biologically effective dose. Also, the effectiveness of AB-100 was dependent upon pH, i.e., a greater effect at pH 5.3 than at pH 7.0. The data showed a quantitative correlation between the concentrations required to decrease the RNA-synthesizing ability of DNA and the in vivo effective doses of the aziridinyl quinones. It may seem that the observed decrease of the ability of DNA to serve as a template in RNA synthesis was due to the "denaturing" effect by the alkylating agents and that it was merely a measure of the denaturation of DNA, a more sensitive one than the decrease of the T_m value (Belman et al. 1964, Grunicke et al. 1965). In the Microsoccus lysoclekikticus RNA-polymerase system, denatured DNA was active as a template and even completely heat-denatured DNA was about one-third as effective as in the native state. However, alkylated DNA showed a similar loss of priming ability (i.e., a decrease to one-third, or less, of the activity of the control) at a state which would correspond to as little as 2 to 3 percent denaturation on the basis of T_m and thermal hyperchromicity values. Thus, the effects of the alkylating agent upon the template activity of DNA are different and apparently more specific in comparison with general denaturation phenomena caused by heat treatment. Chmielewicz et al. (1967) speculated that there may be a more specific inhibition of synthesis of messenger RNA's involved in the synthesis of DNA-replicating enzymes. They also suggested that the DNA polymerase enzyme preparation employed in studies of this type was possibly

a repair enzyme, not a true DNA replicate (Richardson et al. 1964, Schildkraut et al. 1964). Thus it may well be that the studies utilizing cell-free systems or purified enzymes did not reflect the actual sensitivity of DNA synthesis to alkylating agents. Nevertheless, this study shows that the low in vivo concentrations of aziridines do reflect their cytotoxic action.

Evidence showed that the predominant regulation of RNA and DNA synthesis occurs by mechanisms which affect the deoxyribonucleoprotein complex (Stellwagen and Cole 1969, Dahmus and Bonner 1970). It is possible, therefore, that the inhibition of nucleic acid synthesis is caused by a complicated modification of the nucleoprotein structure. Puschendorf et al. (1971) observed a decrease in template activity of chromatin, isolated from Ehrlich ascites tumor cells after treatment with Trenimon, in the presence of RNA polymerase. This inhibition in the template activity of the chromatin was greater after treatment in vivo than after treatment of isolated chromatin with an equivalent concentration in vitro. Deproteinized DNA isolated after in vivo treatment with TEM showed the same inhibition in priming ability as DNA treated in vitro with a corresponding dose. Isolated chromatin treated in vitro exhibited the same sensitivity to the alkylating agent as deproteinized DNA. The authors suggested that the alkylating agent interfered with metabolic reactions, which caused a modification of the biological activity of the nucleoprotein complex. They demonstrated that concentrations of Trenimon which inhibit the template activity of chromatin decreased the acetylation of the acid soluble nuclear proteins. Also, under their experimental conditions, Trenimon treatment (in vivo and in vitro) of chromatin did not lead to an inhibition of its template activity in a DNA system.

Further studies with Trenimon (Wolf et al. 1973) demonstrated that concentrations of the aziridine, which did not affect the biosynthesis of DNA in Ehrlich ascites cells, strongly inhibited the incorporation of (^{3}H)-lysine into histones, whereas (^{3}H)-lysine incorporation into the chromosomal non-histone proteins and total cellular proteins was hardly affected. Apparently, Trenimon was capable of inhibiting the synthesis of DNA and the increase in the amount of histones in the cell nucleus, two processes which are normally tightly coordinated. A suppression of the histone labeling is caused by a direct inhibition of histone synthesis or by a block in the transport of the preformed histones from the cytoplasm into the nucleus. However, a block in the transport of histones seemed unlikely, as the transport of the nonhistone proteins, mainly synthesized in the cytoplasm, was obviously not affected. These studies on the modification of the nucleoprotein show again that aziridines, at extremely low levels, can effect nucleic acid synthesis beyond the sensitivity limits of methods designed to detect base alkylation, phosphate alkylation, and DNA crosslinking.

Grunicke et al. (1973) have shown the modified binding of DNA and protein caused by Trenimon paralleled the inhibition of tumor growth of Ehrlich ascites tumor cells. An alteration of binding in the complex was evident even at the lowest concentration that exerted a measurable effect on cell multiplication. As a result of this modification, which occurred only after treatment with difunctional agents and not after application of monofunctional agents

(Golder et al. 1964, Salser and Balis 1970, Steele 1962), the DNA was resistant to phenol-salt deproteinization procedures. However, Grunicke et al. (1973) showed that methyl methanesulfonate, a monofunctional agent, caused resistance to deproteinization after exposure of tumor cells to drug concentrations at which cell multiplication was inhibited. Since both mono- and poly-functional alkylating agents were capable of rendering DNA resistant to deproteinization, then mechanisms other than crosslinking of DNA and protein by a polyfunctional agent were responsible for this phenomenon.

Reid (1970) demonstrated that treatment of 4-thiouridine with ethylenimine at pH 8 yielded N^4-β-thioethyl-S-β-aminoethyl-cytidine, which was produced by a side-chain rearrangement reaction. In subsequent work, Reid (1971) showed Escherichia coli B tRNA selectively inhibited the acceptance of certain amino acids after exposure to ethylenimine. There was a progressive inactivation of lysine acceptance, and also of histidine, whereas isoleucine and alanine acceptabilities were relatively unaltered. The author stated that, although the inactivation of lysine acceptance appeared to be related kinetically to 4-thiouridine aminoethylation, this correlation may have been coincidental, especially since the selective alkylation of 4-thiouridine in Escherichia coli B tRNA by N-ethylmalemide had little or no effect on the amino acid acceptance activity of the tRNA (Carbon and David 1968). Exposure of the tRNA to (^{14}C)-ethylenimine showed that in addition to 4-thiouridine, another base was aminoethylated. Chromatographic analysis of the tRNA hydrolyzate led Reid (1971) to suggest that the GMP was modified. Proof in the form of molar recovery percentage of the four normal nucleotides was offered as evidence for alkylation of guanine residue; a structure determination of the product was not offered as positive evidence.

CONCLUSIONS

Supporting evidence for possible mechanisms of action of alkylating agents, especially for mustards, appears to involve either a direct or an indirect attack of the DNA. Direct attack of the macromolecule results in base alkylation, phosphate esterification, or crosslinking. Expansion of this hypothesis to include aziridines is not justified by the experimental observations, as few positive results for alkylation by aziridines have been presented; the alkylation theory is derived from results with classical alkylating agents. Mustards have been shown to attack DNA mainly at the N-7 guanine; similar attack by aziridines has been suggested but not supported by isolation and characterization of expected products, i.e., mono- and di-substituted guanines. Lorkiewicz and Szybalski (1961) showed that TEM reacted with pyrimidines DNA precursors in contrast to the accepted theory of N-7 alkylation, but Doskocil (1965) and Lawley and Brookes (1967) claimed that TEM behaved as a typical difunctional mustard by directly attacking the DNA purines. A chromatographic study of a reaction of deoxythymidine with TEM yielded only unreacted deoxythymidine (Lorkiewicz and Szybalski 1961). The authors did not expand this work, and only stated that the mutagenic activity of the product was lost in the attempted isolation. Other workers (Hieble et al. 1973, Lashkova et al. 1973, Ruddon and Mellett 1964, Zybina et al. 1967) have also observed some type of interaction between aziridines and DNA or its components, but the nature of the reactions was unexplained.

As in the preceding cases, isolation and chemical characterization of products from attack by aziridines has evaded all workers. Two explanations for the failure to isolate products can be offered. First, highly reactive intermediate products are destroyed by isolation procedures, i.e., acid hydrolysis and chromatographic systems. For example, Lawley and Brookes (1967) were unable to isolate products when a reaction mixture of TEM and deoxyguanine was applied to an ion exchange resin and eluted with 3N hydrochloric acid. Apparently the compounds were either destroyed by these conditions or irreversibly absorbed on the ion exchange resin. The authors claimed that the aziridinyl moieties reacted with the resin, but this is unlikely as this functional group would have been destroyed by the acid hydrolysis.

A second possibility for failing to isolate products is that a reversible interaction with TEM and DNA components occurred. Then either by hydrolysis or by chromatography procedures the adduct seemingly disappeared and reverted to the initial components, as observed in a reaction of TEM with deoxythymidine (Lorkiewicz and Szybalski 1961). Since the authors did not give recovery percents of initial components, it is impossible to determine whether the products were destroyed or interacted with the adsorbent. One interesting observation in this work showed a doubling of mutagenic activity in Escherichia coli of a reaction product from TEM and deoxythymidine when compared to TEM alone. However, a mixture of TEM and deoxycytidine was completely without mutagenic activity. This type of mutagenic behavior, seemingly enhanced by the presence of deoxythymidine, has also been reported in a study utilizing ethylenimine and thiotepa (Sidorov et al. 1966). A reaction mixture of thymine and ethylenimine was 2.5 times more mutagenic than ethylenimine alone; a product of thiotepa with thymine was also more mutagenic than thiotepa alone. Grinikh (1971) observed an enhancement of the effect of ethylenimine under the action of thymine, which increased the frequency of chromosomal abnormalities.

According to Lorkiewicz and Szybalski (1961), this increased mutagenic activity can be explained as follows. In intercellular, double-stranded DNA, the nucleophilic centers of purine and pyrimidine are hydrogen-bonded and encompassed by nuclear proteins or polyamines. This compact arrangement makes the accessibility of the bases to alkylation very limited for the pyrimidine bases. On the other hand, the intracellular pool of phosphorylated bases might be more accessible to attack. An altered base might then serve as a functional substrate for the enzyme pool, e.g., phosphorylases, pyrophosphorylases and kinases, and subsequently be converted into immediate DNA precursors. Alternatively, direct chemical modification of intercellular precursors might produce analogs immediately available for DNA synthesis. However, there is no evidence for a concept that this modified base, with a presumably different steric configuration, could be incorporated into the cellular deoxyribonucleic acid.

Measurement of high N-7 alkylation by TEM with a tritium assay procedure (Tomasz 1970b) only adds to the dilemma. Why have some workers found high levels of alkylation by TEM when other workers found very low alkylation levels? Why could the products not be isolated? Again these questions can be answered if the possibility is considered that highly unstable or

reversible products are formed after treatment with aziridines. Crosslinking of DNA by polyfunctional aziridines was documented by Alexander et al. (1959), Doskocil (1965), Lawley and Brookes (1967), Chmielewicz et al. (1967), McCann et al. (1971), and Akhtar et al. (1975). However, estimates of the degree of crosslinking as well as its nature, i.e., interstrand and intrastrand contribution, were highly variable and no unified theory of the mechanism of crosslinking was developed.

In general, alkylating agents interact with DNA but to affect the secondary structure of DNA, much higher levels of these agents are needed in vitro to demonstrate the effect observed in vivo. In vivo treatment of cells with low, but lethal, concentrations of these agents does not produce DNA different from untreated systems. Incorporation of ^{14}C-TEM into the DNA fraction of regenerating liver was estimated at 1 mole of drug per mole of DNA polymer (Trams et al. 1961a). This clearly questions the theory of DNA alkylation as the primary mode of action for aziridines. One plausible explanation for the discrepancy could be that the present analytical and radioassay techniques are not sufficiently sensitive to detect very low alkylation levels. Another reason may be that the isolation procedures utilized after treatment of DNA with the alkylating agent are not perfected. Since O-alkylated bases are susceptible to acid hydrolysis in which the base is regenerated, only very mild hydrolysis conditions can lead to successful isolation of unstable interaction products; workers have always used acid hydrolysis in isolation attempts.

Doubts about the N-7 guanine alkylation theory have prompted investigators to explore other theories that would explain the biological effectiveness of aziridines and some nitrogen mustards. One of these concerns the inhibition of nucleic acid synthesis because of the action of these agents on DNA template activity.

Ruddon and Johnson (1968) found that in vivo concentrations of HN2 produced a significant decrease in DNA template activity in a purified RNA polymerase system. Likewise, Chmielewicz et al. (1967) reported inhibitory effects of HN2 and AB-100 on the template function of calf thymus DNA for RNA synthesis; however, the aziridine (AB-100) required activation at slightly acidic conditions. Chmielewicz et al. (1967) suggested that the mechanisms of action involved a specific inhibition of synthesis of messenger RNA's that are involved in the synthesis of DNA-synthesizing enzymes. Grunicke et al. (1973) stated that inhibition of nucleic acid synthesis by alkylating agents may also be caused by an alteration of the nucleoprotein structure since other metabolic inhibitors, e.g., arsenite, iodoacetate, N-ethylmaleimide, and p-chloromercuribenzoate are capable of mimicking the effect of alkylating agents on nucleoproteins. All reagents that proved effective in rendering the DNA resistant to deproteinization by a phenol-salt extraction, a procedure commonly used in DNA isolation, are characterized by a high affinity to SH groups. Although these results do not disprove that polyfunctional alkylating agents caused DNA-protein crosslinks, they encourage other plausible explanations. For instance, it is conceivable that the modified binding is caused by an interference with enzymatic reactions engaged in nucleoprotein metabolism. Doskocil (1965) observed a binding effect of DNA and protein, but the available data are not conclusive and the detailed mechanism of the

modification of nucleoprotein structure remains obscure.

In summary, there is considerable evidence which relates alkylation of DNA to important biological effects. The most significant lesions in DNA have been identified with certainty, but the production of intrastrand and interstrand crosslinks could play a role in explaining the cytotoxicity of polyfunctional alkylating agents. It is unlikely that simple alkylation of DNA can explain all effects observed with alkylating agents. Although most alkylating agents possess similar biological activity, there is a large variation in their selectivity and reactivity. Selectivity of action of an agent is related not only to its alkylating ability, but also to the amount that reaches the target site compared with the amount that reaches other sites of the cells. If it is assumed that DNA is the target site, then the alkylating agent must enter the cell and pass through the cytoplasm to the nucleus before any alkylation of DNA occurs. Differences in cell permeability and in the degree of activation or deactivation of the agent before it reaches DNA, the presence or absence of repair mechanisms, and the cell cycle time influence the sensitivity of each cell.

Clearly, a total reexamination of aziridines and their mode of action is needed. One promising area of research includes an evaluation of the inhibition of template function of DNA for DNA and RNA synthesis by low concentrations of aziridines. Another possibility is a study of the direct or indirect interaction of aziridines with the deoxyribonucleoprotein complex resulting in alteration of the complex. Actually, the combination of these two areas may explain why the concentrations of aziridines required for biological activity are so low. Since there is conflicting evidence for base alkylation, e.g., either attack on purines or pyrimidines, a reevaluation of the proposed direct attack by aziridines on the genetic material is in order. The apparent differences, e.g., in vivo vs. in vitro treatment, observed in measurement of crosslinking ability of various aziridinyl compounds definitely encourage additional research on this phenomenon by highly sensitive methods for detection of low level alkylation. Perhaps the best available method is the fluorometric method in combination with an S_1-endonuclease assay (Lown et al. 1976). Finally, a reexamination of the biological effects of aziridines should be approached only if more stringent controls are placed on reaction conditions and isolation methods. Other considerations should include modifications of established analytical techniques and development of new methods to reexamine all aspects of aziridinyl biological activity.

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